CHAPTER 6

TUDCA protects rat hepatocytes from GCDCA-induced mitochondria-controlled apoptosis via activation of survival pathways

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Abstract

Ursodeoxycholic acid (UDCA) is used in the treatment of cholestatic liver diseases, but its mechanism of action is not well defined yet. The aim of this study is to explore the protective mechanisms of the taurine-conjugate of UDCA (tauroursodeoxycholic acid (TUDCA)) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis in primary cultures of rat hepatocytes. Hepatocytes were exposed to GCDCA, TUDCA, the glyco-conjugate of UDCA (GUDCA) and taurochenodeoxycholic acid (TCDCA). The PI3-kinase (PI3K) pathway and NF-κB were inhibited using LY 294002, and adenoviral overexpression of dominant negative IκB, respectively. The role of p38 and ERK MAP kinase pathways were investigated using the inhibitors SB 203580 and U0 126, and Western blot analysis. Transcription was blocked by actinomycin D. Apoptosis was determined by measuring caspase-3, -9 and -8 activity using fluorimetric enzyme detection, Western blot analysis, immunocytochemistry and nuclear morphology. Our results demonstrated that uptake of GCDCA is needed for apoptosis induction. TUDCA, but not TCDCA and GUDCA, rapidly inhibited, but did not delay, apoptosis at all time points tested. However, the protective effect of TUDCA was independent of its inhibition of caspase-8. Up to six hours pre-incubation with TUDCA prior to GCDCA clearly decreased GCDCA-induced apoptosis. Up to 1.5 hours after exposure with GCDCA, addition of TUDCA was still protective. This protection was dependent on activation of p38, ERK MAP kinases and PI3-kinase pathways, but independent of competition on the cell membrane, NF-κB activation and transcription. In conclusion: TUDCA contributes to the protection against GCDCA-induced mitochondria-controlled apoptosis by activating survival pathways.

Introduction

Cholestatic liver diseases are characterized by accumulation of toxic bile acids, e.g. glycochenodeoxycholic acid (GCDCA), causing damage to hepatocytes and cholangiocytes. Ursodeoxycholic acid (UDCA) is used as a treatment for patients with chronic cholestatic liver diseases. In primary biliary cirrhosis, doses of 13 to 15 mg/kg/day of UDCA decrease serum liver enzymes, improve liver histology, and delay the time to liver transplantation or death for up to 4 years. (1) (2) However, the mechanisms of the beneficial effect of UDCA in these conditions remain unclear. From *in vitro* and *in vivo* studies it is postulated that UDCA protects cholangiocytes against membrane damage induced by hydrophobic bile acids. (3) (4) Furthermore, it has been demonstrated that UDCA stimulates biliary secretion of bile acids and other toxic compounds. (5) (6) In addition, anti-apoptotic effects of UDCA have been described, such as the inhibition of the mitochondrial membrane permeability transition in hepatocytes,(7) (8) leading to prevention of the mitochondrial release of cytochrome c.(9) A recent study has suggested that TUDCA does inhibit apoptosis by preventing the binding of Bax to mitochondria. (10)

Hepatocytes are exposed to many pro-apoptotic compounds. Therefore, anti-apoptotic signalling pathways are important to limit programmed cell death. The anti-apoptotic action of UDCA may in part be due to activation of these anti-apoptotic pathways. A major survival pathway in hepatocytes is the activation of the transcription factor NF-κB. Activation of NF-κB-regulated survival genes causes inhibition of apoptosis. (11) (12). Although we have shown that NF-κB is not activated by bile acids (13), its role in the protection of UDCA against bile acid-induced apoptosis is not clear. Furthermore, other cell survival pathways, like the activation of mitogen-activated protein kinases (MAPK), could be involved in the anti-apoptotic action of UDCA. These kinases are involved in regulation of cell proliferation, differentiation and apoptosis and are comprised of at least three different pathways: ERK, p38 and JNK. (14) Although, it is postulated that inhibition of ERK enhances UDCA-induced apoptosis, (15) little is known about the role of MAP kinases in the protection of taurine-conjugated UDCA against GCDCA-induced apoptosis. Another important survival pathway is the phosphatidylinositol-3 kinase (PI3K) pathway. This kinase cascade results in activation of a number of cellular intermediates of which Akt seems to be one of the most important survival factors. (16) The mechanisms by which PI3K/Akt promote cell survival are diverse and its role in the protection of UDCA against GCDCA-induced apoptosis has not been explored yet. In rats, the taurine conjugate of UDCA predominates compared to glycine-conjugated UDCA.(17;18) Furthermore, TUDCA may be of benefit for patients suffering from primary biliary cirrhosis (19). Therefore, we have investigated the anti-apoptotic actions of TUDCA in primary rat hepatocytes.

Materials and methods

Animals

Specified pathogen-free male Wistar rats (220-250 g) were purchased from Harlan, Zeist, the Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local Committee for Care and Use of Laboratory Animals.

Hepatocyte isolation
Hepatocytes were isolated as described previously (20). Cell viability was consistently more than 90 % as determined by trypan blue exclusion. Isolated hepatocytes were cultured in William's medium E (Life Technologies Ltd., Breda, The Netherlands) supplemented with 50 μg/ml gentamycin (BioWhittaker, Verviers, Belgium) without the addition of hormones or growth factors. During the attachment period (4 hours) 50 nmol/L dexamethasone (Sigma, St Louis, MO) and 5 % fetal calf serum (Life Technologies Ltd.) were added to the medium.

Experimental design
Experiments were started twenty-four hours after isolation. Hepatocytes were exposed to 50 μmol/L GCDCA (Calbiochem, La Jolla, CA), 50 μmol/L TUDCA (Calbiochem), 50 μmol/L glyco-ursodeoxycholic acid (GUDCA, Calbiochem), or 50 μmol/L TCDCA (Calbiochem) for 4 hours or the indicated time period. In some experiments, hepatocytes were exposed to 20 ng/ml recombinant mouse tumor necrosis factor α (TNFα, R&D Systems, Abingdon, United Kingdom) or a mixture of cytokines as described previously (11). Signal transduction or apoptosis pathways were specifically inhibited with the following compounds: 50 μmol/L of caspase-8 inhibitor Ac-IETD-CHO or the caspase-3 inhibitor Ac-DEVD-CHO (Biomol, Plymouth Meeting, USA), 10 μmol/L of the p38 inhibitor SB 203580 (Biomol), 10 μmol/L of the ERK1/2 inhibitor U0126 (Promega, Madison, USA), 50 μmol/L PI3-kinase inhibitor LY 294002 (Sigma-Aldrich), and 200 ng/ml of the transcriptional inhibitor actinomycin-D (Roche Diagnostics, Almere, The Netherlands). All inhibitors were added 30 minutes prior to bile acids or cytokines. Hepatocytes received adenovirus (MOI of 10) fifteen hours prior to exposure of bile acids. Each experimental condition was performed in triplicate wells. Each experiment was performed at least three times, using hepatocytes from different isolations.Cells were harvested at the indicated times after the addition of bile acids as described previously (11).

HepG2 cell experiments
The human hepatoma cell line HepG2 and a stable derivative expressing rNtcp were cultured as described before (21;22). Cells were incubated with indicated amounts of GCDCA or 1 μg/ml anti-Fas antibody (clone nr. 7C11; Immunotech, Marseille, France) for 4 hours followed by harvesting in hypotonic cell lysis buffer (11).

Adenoviral constructs
Adenoviral constructs have been described previously (23).

Caspase-3 and caspase-8 enzyme activity assay
Hepatocytes were scraped and cell lysates were obtained by three cycles of freezing (-80 °C) and thawing (37 °C) followed by centrifugation for 5 minutes at 13.000 g. Caspase-3 and caspase-8 enzyme activities were assayed as described previously (11,13).

Nuclear staining

Morphological features of apoptotic nuclei were demonstrated with acridine orange. Cells were seeded on glass coverslides and treated as indicated. These coverslides were fixed in methanol for 5 minutes, air-dried and rinsed twice in phosphate buffer saline before incubating in acridine orange (1:1000) for 15 minutes in the dark. Fluorescent nuclei were visualized using a Leica confocal laser scanning microscope.

Immunocytochemistry and uptake of fluorescent bile acids

Analysis of active caspase-9 was performed on hepatocytes cultured on coverslips and exposed to 50 μmol/L bile acids for 4 hours. Coverslips were washed in phosphate buffer saline, fixed in 4 % paraformaldehyde for 10 minutes followed by incubation in 1 % Triton-X100 for 5 minutes. Antibody against active caspase-9 was used at a dilution of 1:50 for 30-60 minutes. Fluorexein isothiocyanate-conjugated goat-anti-rabbit Ig (Molecular Probes, Eugene, Oregon, USA) was added at a dilution of 1:600 for 45 minutes.

The uptake of bile acids was demonstrated using the fluorescent bile acid cholyl lysyl fluorescein (CLF) (24). Cells were incubated with 2 μmol/L CLF at 37 °C for 15 minutes. All slides were evaluated on a Leica confocal laser scanning microscope.

Western blot analysis

Western blot analysis of cell lysates was performed using polyclonal rabbit antibodies against cleaved caspase-9 and phosphorylated p38 MAP, and monoclonal antibody against phosphorylated ERK1/2 (p44/42) MAP kinase (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. Hepatocytes exposed to 50 μmol/L deoxycholic acid (DCA) served as positive control for phophorylated ERK1/2(25). In addition, activated neutrophil extracts (kindly provided by Dr. Gwenny Fuhler, Department of Haematology, University Hospital Groningen) were used to confirm detection of phosphorylated ERK1/2 MAP kinase. For the detection of caspase-9 and phospho-p38, horse radish-peroxidase conjugated swine-anti-rabbit Ig was used as a secondary antibody at a dilution of 1:2000. Phospho-ERK1/2 was detected with horse radish-peroxidase rabbit-anti-mouse Ig (1:2000). Each lane contained the lysate of 150000 cells. Equal loading was demonstrated by Ponceau-S staining. After Western blot analysis of phosphorylated p38 and ERK1/2 MAP kinases, blots were stripped using 0.1 % SDS at 65 °C for 30 minutes and incubated with 1:1000 antibody against total p38 MAP kinase or total ERK1/2 MAP kinase (Santa Cruz Biotechnology, Santa Cruz, USA). Western blot analysis for iNOS was performed as described before (26).

Statistical analysis

Results are presented as the mean of at least 3 independent experiments ± standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant**.**

Results

Ntcp is required for GCDCA-induced mitochondria-controlled apoptosis

To investigate the protective mechanisms of TUDCA against GCDCA-induced apoptosis, we first determined whether GCDCA needs to be taken up by cells to induce apoptosis. Previous studies have postulated death receptor activation by toxic bile acids (27-29). However, strong distinction between the role of the bile acid uptake transporter Ntcp and death receptor-mediated apoptosis has not been made yet. Recently, we have shown that GCDCA induced apoptosis in a mitochondrial-controlled manner in primary rat hepatocytes, which was FADD-independent. (13) In the present study, we examined whether Ntcp is required for GCDCA-induced apoptosis. For this purpose, we exposed Ntcp-negative and Ntcp-positive HepG2 cells(21) to different concentrations of GCDCA. Both cell lines do express Fas death receptor. Anti-Fas antibody served as positive control. As shown in Figure 1A, only HepG2 cells expressing Ntcp on their cell membrane are sensitive to GCDCA-induced apoptosis, which increased with increasing amounts of GCDCA. In contrast, anti-Fas antibody induced apoptosis in both Ntcp-positive and Ntcp-negative cell lines to the same extent. These data indicate that GCDCA first needs to be taken up by Ntcp before the onset of apoptosis and that ligand-dependent death receptor activation is not involved.

To exclude HepG2-specific artifacts, primary hepatocytes were exposed to GCDCA at 24 hours and 72 hours after isolation. Ntcp was expressed at high levels 24 hours after isolation (data not shown) but decreased in hepatocytes maintained in primary cultures for 72 hours, thereby confirming previous data.(30) In addition, uptake of bile acids in these hepatocytes decreased in time, as shown with the fluorescent bile acid CLF (Fig 1C). Accumulation of CLF was only detected in bile canaliculi of hepatocytes cultured for 24 hours, as previously reported.(24) At this time point, GCDCA strongly induced caspase-3 activity. In contrast, GCDCA did not induce caspase-3 activity anymore in hepatocytes cultured for 72 hours (Fig 1B). Induction of caspase-3 activity by TNF-α in the presence of actinomycin-D remained unaffected. All together, these data provide evidence that Ntcp is required for GCDCA-induced apoptosis in primary hepatocytes.

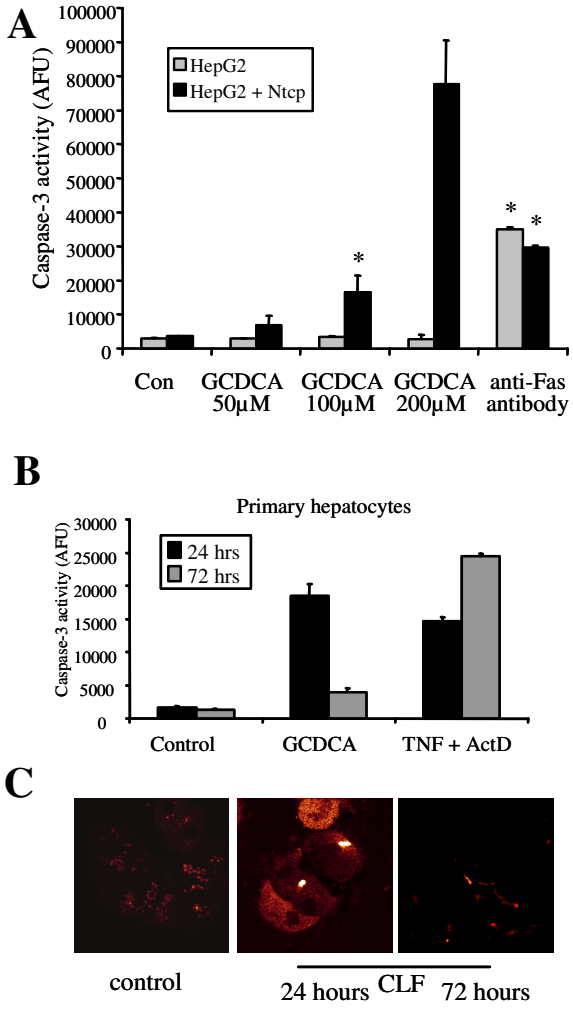

Figure 1. Ntcp is required for glycochenodeoxycholic acid (GCDCA)-induced apoptosis. (A) Ntcp positive and negative HepG2 cells were treated with different amounts of GCDCA and 1 µg/µl anti-Fas antibody (positive control) for 4 hours. Only in Ntcp-positive HepG2 cells, caspase-3 activity increased with increasing amounts of GCDCA. *P<0.05 for GCDCA 100 µM and anti-Fas antibody vs. control. (B) Caspase-3 activity in primary rat hepatocytes exposed to 50 µmol/L GCDCA or 20 ng/ml TNF-α in the presence of actinomycin-D (ActD) at 24 hours and 72 hours after isolation. (C) Uptake of bile acids demonstrated by fluorescence microscopy. Primary hepatocytes were exposed to 2 µmol/L of the fluorescent bile acid cholyl lysyl fluorescein (CLF) for 15 minutes. Accumulation is observed in the bile canaliculus between adjacent hepatocytes at 24 hours but not 72 hours after isolation. (Original magnification is 400x).

TUDCA inhibits but does not delay GCDCA-induced caspase-3 activity and apoptotic nuclear morphology

GCDCA-induced caspase-3 activity in hepatocytes peaks around 4 hours. (13) Therefore, at this time-point, we investigated the effect of TUDCA on GCDCA-induced caspase-3 activity in primary hepatocytes. TUDCA itself did not induce caspase-3 activation (Fig 2a) confirming previous data. (13) TUDCA, but not TCDCA and GUDCA, inhibited GCDCA-induced caspase-3 activity for 70 % as shown in Figure 1a. A concentration-dependent curve displayed that the minimal concentration exerting the maximal protective effect is 50 µM of TUDCA (Fig 2b). To demonstrate that TUDCA inhibits but does not delay GCDCA-induced caspase-3 activity, a time course study was performed. Two to 15 hours after addition of GCDCA + TUDCA, caspase-3 activity was inhibited significantly at all time-points (Fig 2c). Nuclei staining was performed with acridine orange confirming that GCDCA-induced activation of caspase-3 activity results in apoptosis. Nuclear fragmentation and condensation were observed 4 hours after the addition of GCDCA, which increased after 8 hours and persisted up to 15 hours. After 8 hours, 30 to 40 percent of hepatocytes displayed apoptotic nuclei, which was inhibited in the presence of TUDCA to 5 percent (Fig 2d). Cytokine-

exposed hepatocytes in which transcription was blocked with actinomycin-D served as positive control.

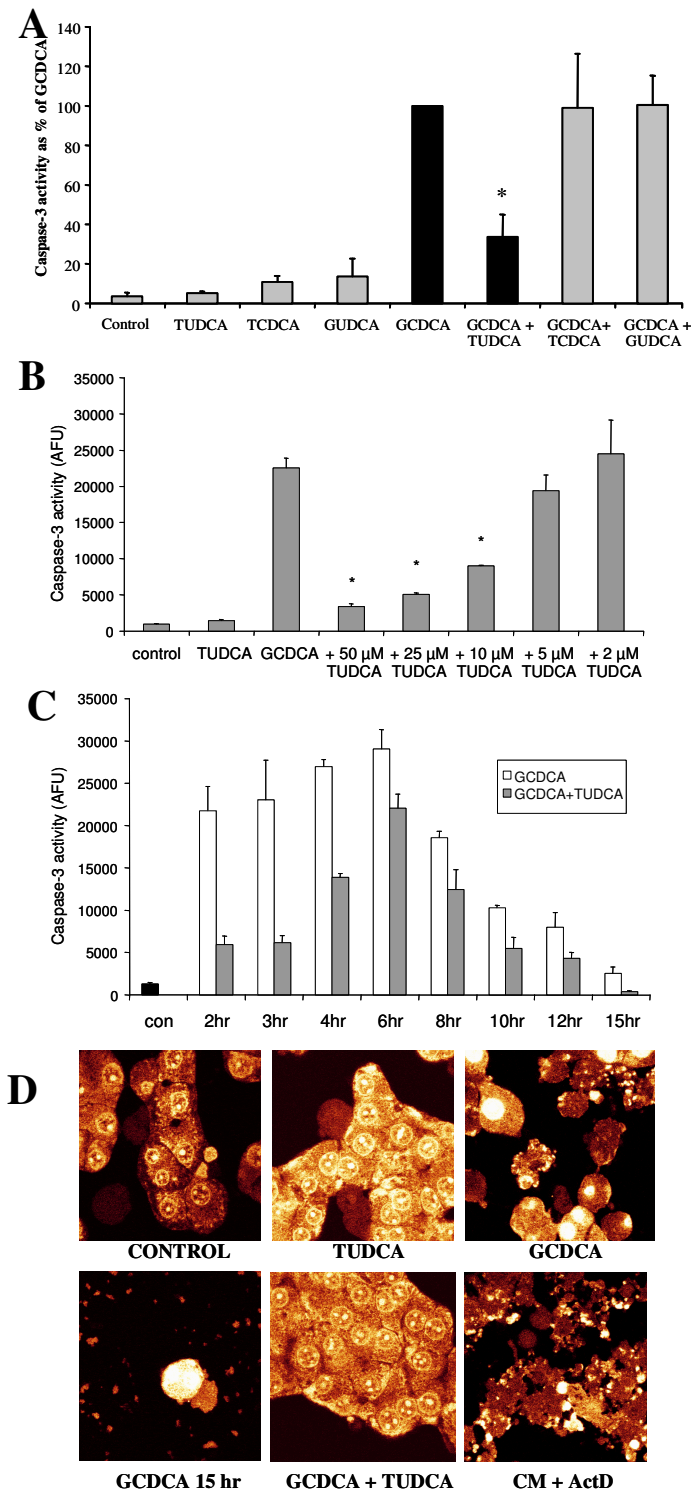

Figure 2. Tauroursodeoxycholic acid (TUDCA), but not taurochenodeoxycholic acid (TCDCA) and glycoursodeoxycholic acid (GUDCA), inhibits glucochenodeoxycholic acide (GCDCA)-induced caspase-3 activity and nuclear fragmentation. (A) Primary rat hepatocytes were stimulated for 4 hours with 50 µmol/L of GCDCA, 50 µmol/L of TUDCA, 50 µmol/L of TCDCA, 50 µmol/L of GUDCA, or a combination thereof.. Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition. *P < 0.05 for GCDCA + TUDCA vs. GCDCA alone. (B) Caspase-3 activity. Primary rat hepatocytes were stimulated for 4 hours with 50 µmol/L of GCDCA and different amounts of TUDCA, as indicated in the figure. *P<0.05 for GCDCA + 50, 25, and 10 µM TUDCA vs. GCDCA alone. (C) Time course study. TUDCA (50 µmol/L) significantly inhibits GCDCA-induced caspase-3 activity at all indicated time-points. Representative data of 3 independent experiments are shown.. (D) Nuclear morphology in hepatocytes as determined by acridine orange staining. Cells were treated for 8 hours as indicated in the figure. Treatment with 50 µmol/L GCDCA induces nuclear condensation and fragmentation, which persists for at least 15 hours and is blocked with 50 µmol/L TUDCA. Hepatocytes treated with cytokinemix (CM) + actinomycin-D (ActD) for 15 hours served as positive control

The protective action of TUDCA depends on the inhibition of caspase-9 activation

Because we previously noticed that GCDCA induces apoptosis in a mitochondria-controlled manner (13), the effects of TUDCA on caspase-9 and caspase-8 were investigated. As shown in Figure 3, TUDCA also prevented GCDCA-induced activation of caspase-9. Both Western blot (Fig 3A) and immunocytochemistry (Fig 3B) demonstrated this effect.

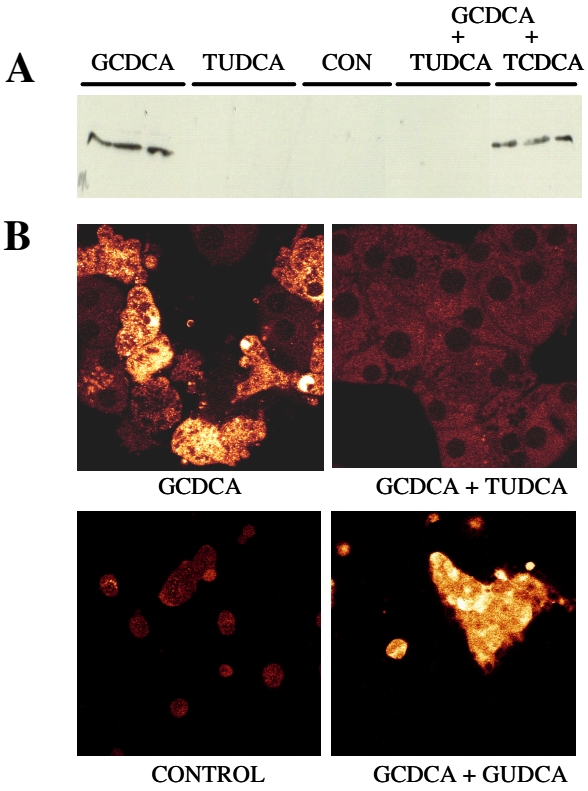

Figure 3. Tauroursodeoxycholic acid (TUDCA), but not taurochenodeoxycholic acid (TCDCA) or glycoursodeoxycholic acid (GUDCA), prevents glycochenodeoxycholic acid (GCDCA)-induced activation of caspase-9. Primary rat hepatocytes were exposed for 4 hours to 50 µmol/L of GCDCA, 50 µmol/L of TUDCA or both, plus TCDCA or GUDCA. (A) Western blot analysis on cell lysates for active caspase-9. (B) Immunocytochemistry for active caspase-9 (original magnification is X 400). AFU, arbitrary fluorescent units

Although TUDCA inhibited GCDCA-induced caspase-8 activity for 50 % (Fig 4B), we have previously shown that caspase-8 inhibition does not inhibit GCDCA-induced caspase-3 activity. (13) In contrast, the caspase-3 inhibitor Ac-DEVD-CHO and the caspase-9 inhibitor Ac-LEHD-CHO inhibited GCDCA-induced activation of both caspase-3 (Fig 4A) and caspase-8 (Fig 4B). These data demonstrate that inhibition of caspase-9 is more important in the TUDCA-mediated protection against GCDCA-induced apoptosis than caspase-8 inhibition.

In contrast to TUDCA, TCDCA and GUDCA did not inhibit GCDCA-induced caspase-9 Activity (Fig 3) and caspase-8 activity (Fig 4B).

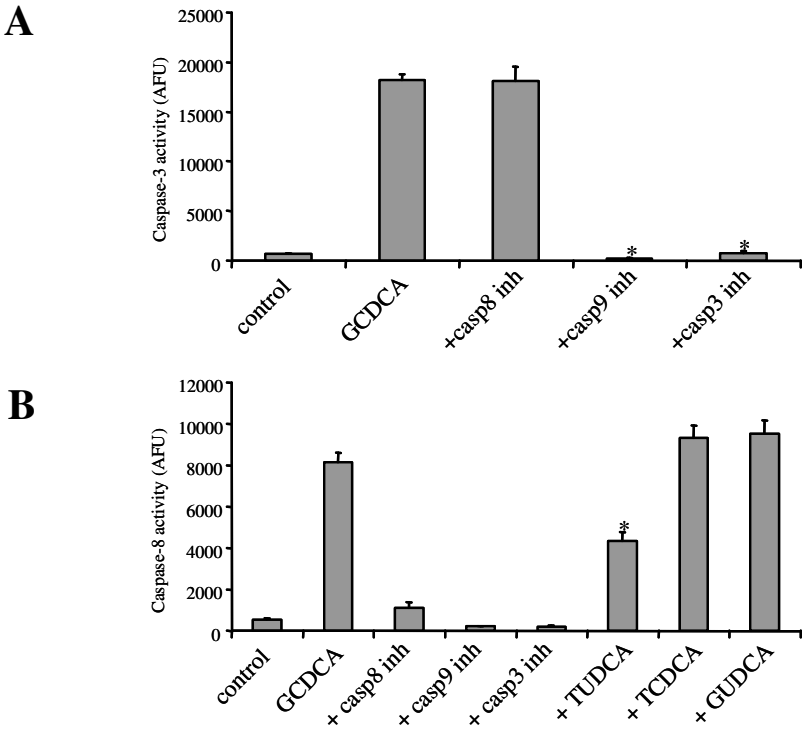

Figure 4. Caspase-3 and caspase-8 activation in glycochenodeoxycholic acid (GCDCA)-exposed primary rat hepatocytes in the presence of bile acids and caspase inhibitors. Representative data of 3 independent experiments are shown with n = 3 per condition. (A) Caspase-3 activity. Cells were treated for 4 hours with 50 µmol/L of (GCDCA) plus 50 µmol/L caspase-8 inhibitor, 50 µmol/L caspase-9 inhibitor, or 50 µmol/L caspase-3 inhibitor. *P<0.05 for GCDCA + caspase-9 inhibitor and GCDCA + caspase-3 inhibitor vs. GCDCA. (B) Caspase-8 activity. Cells were treated for 4 hours with 50 µmol/L of GCDCA plus 50 µmol/L tauroursodeoxycholic acid (TUDCA), 50 µmol/L taurochenodeoxycholic acid (TCDCA), or 50 µmol/L glycoursodeoxycholic acid (GUDCA), 50 µmol/L caspase-8 inhibitor, 50 µmol/L caspase-9 inhibitor, or 50 µmol/L caspase-3 inhibitor. *P<0.05 for GCDCA + TUDCA vs. GCDCA. AFU, arbitrary fluorescent units.

TUDCA does not compete with GCDCA for uptake at the cell membrane

Next, we investigated whether TUDCA needs to be present at the same time with GCDCA, before GCDCA or after the addition of GCDCA, to exert its protective effect. For this purpose, we pre-incubated hepatocytes with TUDCA for 9, 6 and 3 hours, washed these cells and exposed them to GCDCA in fresh medium for 4 hours. Six and 3 hours pre-incubation with TUDCA significantly inhibited GCDCA-induced caspase-3 activity for 50 % (Fig 5A). Pre-incubation with TUDCA for 3 hours also inhibited GCDCA-induced caspase-9 activation (Fig 5B). Furthermore, exposure to TUDCA up to 90 min after the addition of GCDCA still exerted protection against GCDCA-induced apoptosis (Fig 5C). At this time point, GCDCA-induced apoptosis had not reached its maximum yet (Fig 5D), confirming previous results.(13) These data indicate that the protective effect of TUDCA is not due to competition with GCDCA for uptake at the cell membrane. In addition, these data imply that the anti-apoptotic actions of TUDCA are very rapidly induced since simultaneous addition or addition of TUDCA after GCDCA is still able to prevent apoptosis in these cells. This could mean that signalling cascades are activated by TUDCA.

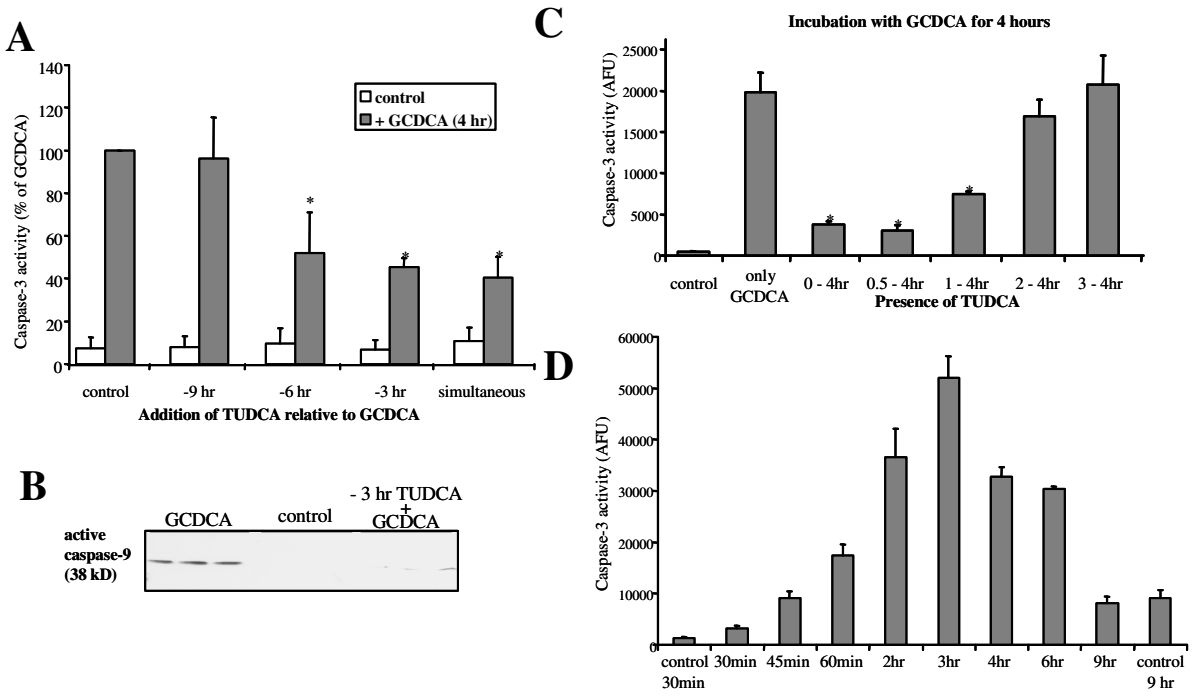

Figure 5. Tauroursodeoxycholic acid (TUDCA) does not compete with glycochenodeoxycholic acid (GCDCA) for uptake at the cell membrane. (A) Pre-incubation with TUDCA inhibits GCDCA-induced apoptosis. Primary rat hepatocytes were exposed to 50 µmol/L of TUDCA for 9 (-9 hr), 6 (-6 hr) and 3 (-3 hr) hours after which cells were washed and exposed to 50 µmol/L of GCDCA for 4 hours. Cells were also incubated for 4 hours with GCDCA alone or with simultaneous addition of TUDCA (simultaneous). Caspase-3 activity is presented as percentage of GCDCA alone. *P<0.05 for -6 hours, -3 hours and simultaneous incubation of TUDCA + GCDCA vs. GCDCA alone (control). (B) Western blot analysis for active caspase-9 on cell lysates of hepatocytes pre-incubated for 3 hours with TUDCA followed by addition of GCDCA in refreshed medium for 4 hours. (C) Caspase-3 activity. Primary rat hepatocytes were incubated with 50 µmol/L of GCDCA for 4 hours (only GCDCA). Simultaneously (0-4 hr) or half an hour (0.5-4 hr), 1 hour (1-4 hr), 2 hours (2-4 hr), or 3 hours (3-4 hr) after the addition of GCDCA, 50 µmol/L TUDCA was added. All cells were harvested 4 hours after the addition of GCDCA. Representative data of 3 independent experiments are shown with n = 3 per condition. *P<0.05 for (0-4 hr) of TUDCA, (0.5-4 hr) of TUDCA, (1-4 hr) of TUDCA vs. only GCDCA. (D) Time course study of (50 µmol/L) GCDCA-induced caspase-3 activity. Representative data of 3 independent experiments are shown with n = 3 per condition.

NF-κB is not involved in the protection of TUDCA against GCDCA-induced apoptosis

Previous results demonstrated that activation of the transcription factor NF-κB resulted in the transcription of survival genes protecting hepatocytes against apoptosis (11) (12). However, TUDCA does not activate NF-κB and does not induce the expression of NF-κB-regulated anti-apoptotic genes (13). Because NF-κB can be activated indirectly, we investigated the role of NF-κB activation in relation to the anti-apoptotic mechanisms of TUDCA. Therefore, primary hepatocytes were infected with recombinant adenovirus expressing dominant negative IκB preventing NF-κB activation. Functionality of this virus was demonstrated by EMSA and sensitizing hepatocytes to cytokine-induced apoptosis (data not shown). As presented in Figure 6, inhibition of the NF-κB survival pathway does not significantly reduce the protective effect of TUDCA against GCDCA-induced caspase-3 activity.

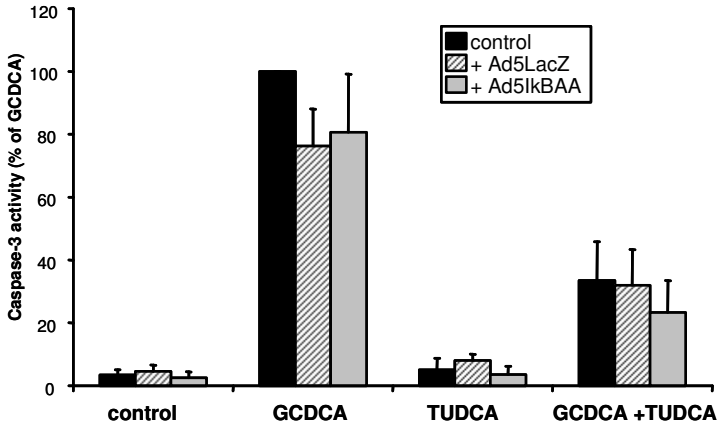

Figure 6. NF-κB is not involved in the protection of Tauroursodeoxycholic acid (TUDCA) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis. Primary rat hepatocytes were exposed for 4 hours to 50 µmol/L of GCDCA, 10 µmol/L of TUDCA or both, 15 hours after receiving 10 plaque-forming units/cell of recombinant adenovirus inhibiting NF-κB activation (Ad5IkBAA). Ad5LacZ served as control virus. Protection of TUDCA against GCDCA-induced caspase-3 activity did not change significantly. Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition.

Anti-apoptotic action of TUDCA depends on the activation of p38 MAP kinase, ERK MAP kinase, and PI3 kinase, whereas gene transcription is not involved

To investigate whether PI3 kinase and MAP kinases pathways are involved in the anti-apoptotic effects of TUDCA, specific inhibitors of PI3K (LY 294002), p38 MAPK (SB 203580) and ERK1/2 MAP kinase (U0126) were used. Dimethyl sulfoxide was used as a solvent for inhibitors but did not have an effect itself (data not shown). Experiments with inhibitors in control hepatocytes and in hepatocytes exposed to TUDCA or GCDCA were included as well. Hepatocytes exposed to inhibitors of MAPK pathways in the absence of bile acids demonstrated caspase-3 values around control level (Fig 7A). Caspase-3 activity increased slightly when PI3 kinase was blocked in control hepatocytes, and this effect was enhanced in combination with MAPK inhibitors. This pattern was similar for hepatocytes exposed to inhibitors in the presence of TUDCA (Fig 7A).

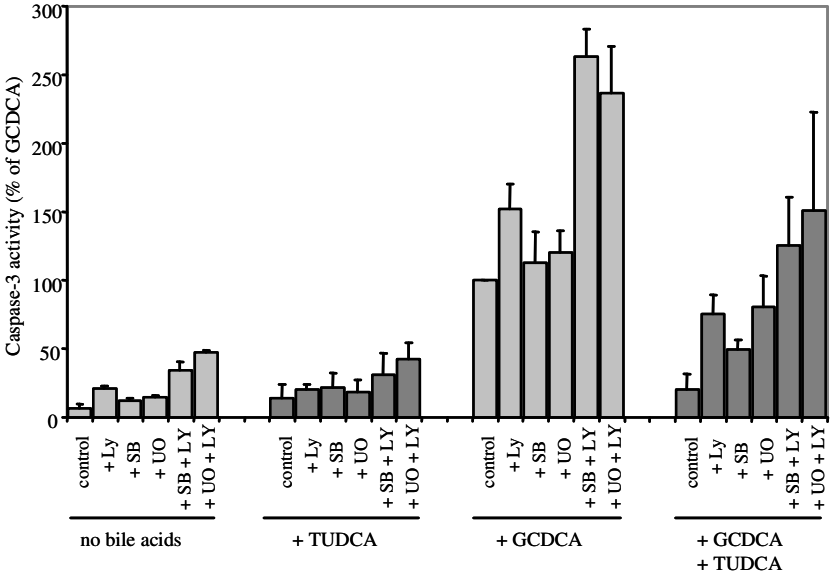

Figure 7. Tauroursodeoxycholic acid (TUDCA) protects against glycochenodeoxycholic acid (GCDCA)-induced apoptosis by activation of the p38 mitogen-activated protein dinase (MAPk), ERK1/2 MAPK and the PI3K pathway. (A) Caspase-3 activity in primary rat hepatocytes treated as indicated in the figure with 50 µmol/L of GCDCA, 50 µmol/L of TUDCA or both with or without inhibitors of ERK MAPK (10 µmol/L of U0126; UO), p38 MAPK(10 µmol/L of SB 203580; SB), PI3K (50 µmol/L of LY 294002; LY) or a combination thereof. Caspase-3 activity is presented as percentage of GCDCA alone. Data represent mean of at least 3 independent experiments with n = 3 per condition. ▼P<0.05 for control + LY, control + SB + LY, and for control + UO + LY vs. control. ▲P<0.05 for TUDCA + SB+LY vs TUDCA and for TUDCA + UO+LY vs TUDCA. ✕P<0.05 for GCDCA + LY vs GCDCA and for GCDCA + SB+LY vs. GCDCA and for GCDCA + UO+LY vs. GCDCA. ■P<0.05 for GCDCA+TUDCA vs. GCDCA. #P<0.05 for GCDCA+TUDCA + inhibitors vs. GCDCA+TUDCA

GCDCA-induced apoptosis was slightly, but not significantly, enhanced with inhibitors of p38 or ERK MAP kinases (Fig 7A). In contrast, inhibition of PI3K pathway aggravated GCDCA-induced caspase-3 activity significantly. In the presence of both MAPK and PI3K inhibitors, exposure to GCDCA significantly increased caspase-3 values compared to GCDCA alone. The protective effect of TUDCA against GCDCA-induced apoptosis was partially, but significantly abolished by inhibition of p38 MAPK pathway (Fig 7A).
In support, TUDCA activated p38, which was blocked by SB 203580 as shown in Figure 7B. Western blot demonstrated equal presence of total p38 MAP kinase in all lanes (Fig 7B). Blocking of the PI3 kinase survival cascade resulted in abrogation of TUDCA protection against apoptosis as well (Fig 7A). The protective effect of TUDCA against GCDCA-induced apoptosis was completely abolished when both p38 MAP kinase and PI3 kinase pathways were blocked (Fig 7B).

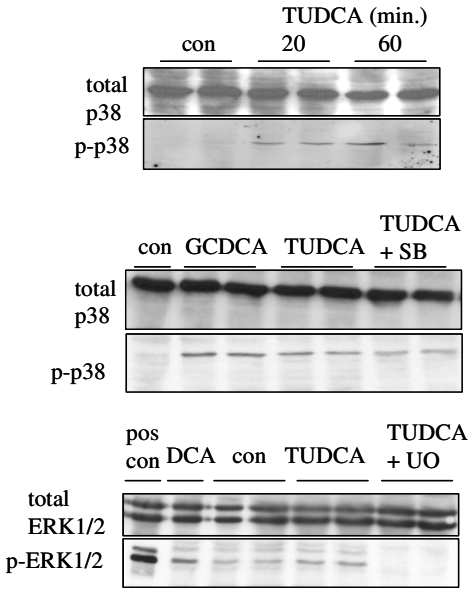

Figure 7 (B+C). Tauroursodeoxycholic acid (TUDCA) protects against glycochenodeoxycholic acid (GCDCA)-induced apoptosis by activation of the p38 mitogen-activated protein dinase (MAPk), ERK1/2 MAPK and the PI3K pathway. (B) Western blot analysis for phosphorylated p38 MAPK in control lysates (con) and cell lysates of TUDCA-exposed hepatocytes (20 and/or 60 minutes) with or without p38 MAPK inhibitor (SB). The same blots for total p38 MAP kinase are presented. (C) Western blot analysis for phosphorylated ERK1/2 MAPKin cell lysates of TUDCA-exposed hepatocytes (30 minutes) with and without 10 µmol/L of ERK1/2 inhibitor U0126, and control lysates (con). Positive control was 50 µmol/L of deoxycholic acid (DCA) for ERK1/2 phosphorylation in hepatocytes, whereas an activated neutrophil extract was included for Western blot detection of ERK1/2 (pos. con). Upper panel presents same blot for total ERK1/2 MAPK.

Next, the ERK1/2 MAP kinase was investigated. As shown in Figure 7A, specific inhibition of ERK1/2 MAP kinase using U0126 significantly prevented the protective effect of TUDCA against GCDCA-induced apoptosis. In addition, Western blot demonstrated that TUDCA activates ERK1/2, which can be blocked with U0126 (Fig 7C). The bile acid deoxycholic acid (DCA) was included as positive control for hepatocytes, which was confirmed with a neutrophil extract displaying high level of phopho-ERK1/2. U0126 also inhibited DCA-mediated ERK1/2 phosphorylation (data not shown). Total ERK1/2 MAP kinase was equally present in all lanes (Fig 7c). Inhibition of both ERK1/2 MAP kinase and PI3 kinase abolished the protective effect of TUDCA against GCDCA-induced apoptosis completely (Fig 7A).

Finally, the role of trancription in the protection of TUDCA against bile acid-induced apoptosis was studied. Inhibition of transcription using actinomycin D, at a dose sensitizing hepatocytes to cytokine-induced apoptosis (Fig 2D) had no influence on the protective effect of TUDCA (Fig 8). Blocking of transcription was confirmed with Western blot for cytokine-induced transcription of inducible nitric oxide synthase (iNOS) (Fig 8 inset) (11).

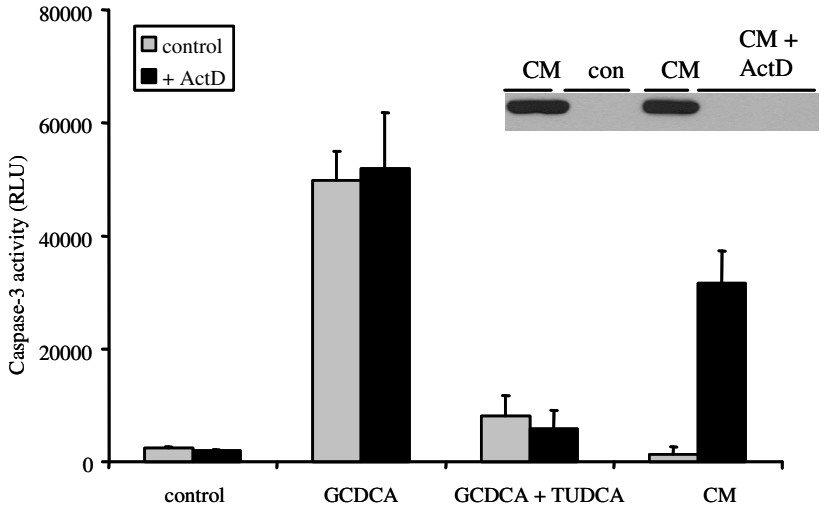

Figure 8 Transcription-independent protection of tauroursodeoxycholic acid (TUDCA) against glycochenodeoxycholic acid (GCDCA)-induced apoptosis. Caspase-3 activity in rat hepatocytes treated with 50 µmol/L GCDCA, 50 µmol/L of TUDCA or both with or without 200 ng/ml of transcription inhibitor actinomycin-D (ActD) for 4 hours. ActD did not significantly change the protective effect of TUDCA. Cytokine-exposed hepatocytes (CM) for 6 hours served as positive control for ActD. Inset: 200 ng/mL ActD blocks transcription. Western blot for cytokine (CM)-induced expression of inducible nitric oxide synthase (iNOS), 11 hours after exposure. See Materials and Methods for details. RLU, relative light units.

Discussion

In this study, we investigated the protective mechanisms of taurine-conjugated UDCA against GCDCA-induced apoptosis in primary rat hepatocytes. We have previously shown that GCDCA induces apoptosis in a mitochondria-dependent manner, in which FADD is not involved and caspase-8 activation is not initially required. (13) In the present study, we investigated this further by blocking caspase-9. Indeed, peptide inhibitors of caspase-9 and caspas-3 blocked caspase-8 activation. Although these caspase inhibitors could have overlapping inhibitory effects, previous results with the human homologue of Inhibitor of Apoptosis protein1 (HIAP1) demonstrated that overexpression of HIAP1 inhibited GCDCA-induced apoptosis. HIAP1 exclusively inhibits caspase-3 and caspase-9 activation, but not caspase-8 (31). These data indicate that GCDCA induces apoptosis in a mitochondria-controlled manner.

In the present study, we demonstrated that TUDCA inhibits, but does not delay GCDCA-induced caspase-9 and caspase-3 activity and the formation of apoptotic nuclei. Furthermore, we have shown that Ntcp is required for GCDCA-induced apoptosis in primary hepatocytes, indicating that ligand-dependent death receptor activation is not likely to occur. This report demonstrates that the anti-apoptotic action of TUDCA against GCDCA-induced apoptosis is not due to a direct competitive effect on the cell membrane. For example, TUDCA could compete with GCDCA for uptake by the bile acid importer Ntcp, thus preventing the uptake of pro-apoptotic GCDCA into hepatocytes. Pre-incubation for several hours with TUDCA followed by removal of TUDCA inhibits GCDCA-induced caspase-3 and caspase-9 activity,

indicating the activation of survival pathways. Nine hour pre-incubation with TUDCA does not protect cells against GCDCA, likely because of quenching of survival signals.
Overall, TUDCA appears to protect mitochondria from GCDCA-induced injury by preventing GCDCA-induced caspase-9 activation rather than by preventing death receptor-mediated apoptosis.
The inhibitory effect on GCDCA-induced apoptosis is exclusively exerted by TUDCA. Our results demonstrate that taurine-conjugated CDCA does not inhibit GCDCA-induced caspase-3, caspase-9 and caspase-8 activity. This is in contrast to others showing inhibition of Fas-mediated caspase-8 activation by TCDCA-induced PI3-kinase. (32) However, the regulation of cell survival and apoptosis in a hepatoma cell line and primary non-transformed hepatocytes differs and this may explain the different results.
GUDCA could be clinically relevant since in humans, contrary to rats, glycine-conjugated UDCA is much more abundant compared to taurine-conjugated UDCA (17;18). On the other hand, TUDCA has been reported to be beneficial in humans.(19) Nevertheless, TUDCA abundance in rats could explain why GUDCA does not inhibit GCDCA-induced apoptosis in rat hepatocytes *in vitro*. Primary human hepatocytes should give more information about the protective effect of taurine- and glycine-conjugated UDCA against bile acid-induced apoptosis.
Our data demonstrate that the p38 and ERK MAP kinase pathways and the PI3 kinase pathway are involved in the protection of TUDCA against GCDCA-induced apoptosis. Because blocking of both PI3 and MAP kinases exaggerates caspase-3 activity compared to blocking a single pathway, these pathways are partly redundant. Moreover, inhibition of the PI3 kinase pathway alone or in combination with MAP kinase inhibitors aggravates GCDCA-induced apoptosis and sensitizes hepatocytes slightly to TUDCA. Indeed, we have demonstrated that in addition to TUDCA (Fig 7B), GCDCA activates p38 and ERK MAP kinases (data not shown). These results are important for the interpretation of the protective effects of TUDCA. Blocking of protective kinases induces higher caspase-3 activity in GCDCA-exposed hepatocytes compared to GCDCA + TUDCA-exposed hepatocytes, implying additional protective effects of TUDCA, e.g. direct alterations of the mitochondrial membrane environment. Evidence for this was given recently by showing that TUDCA stabilizes the lipid and protein structure of mitochondrial outer membranes, thus inhibiting Bax binding to the outer membrane (10).
Because simultaneous addition of TUDCA and GCDCA, or addition of TUDCA after GCDCA blocks apoptosis in hepatocytes, TUDCA very rapidly exerts its protective effect. Indeed, no transcription or NF-κB activation is needed for protection, indicating that survival signaling involves post-translational mechanisms exerted within 30 minutes as shown by Western blot (Fig 7B, C). TUDCA protection against GCDCA-induced apoptosis can only be exerted up to 1,5 hours after GCDCA addition. An explanation could be that in this time frame, GCDCA-induced apoptosis has not reached its maximum yet, whereas after 2 hours, apoptosis has proceeded beyond the point that TUDCA can be protective. Further evidence for rapid anti-apoptotic mechanisms of TUDCA is inferred from our previous results using cytokine-mediated protection against GCDCA. Cytokines do not protect against bile acid-

induced apoptosis after the addition of GCDCA, since their protective mechanism depends on NF-κB-mediated transcription with a time frame of hours instead of minutes (13).
Our PI3K data supports other reports, although in these studies unconjugated UDCA was found to be apoptotic in itself (15). Activation of Akt protects against apoptosis via several mechanisms, including the phosphorylation of the pro-apoptotic Bcl-2 family member Bad, which can no longer associate with and inhibit anti-apoptotic Bcl-XL. (33) (34) Akt activation also results in the phosphorylation and inactivation of caspase-9 (35) and may suppress pro-apoptotic Bax translocation to the mitochondria.(36) It is reported that bile acids activate the ERK1/2 MAP kinase pathway via activation of the EGF receptor. (15) (25;37;38) The exact mechanism is still unclear, but mitochondrial-derived reactive oxygen species may be involved as recently suggested(37;39), and TUDCA prevents the generation of ROS.(10) The inhibition of Bax relocation to the mitochondria could also be mediated by ERK1/2 MAP kinase.(40) Since TUDCA prevents Bax-induced membrane perturbation(10), TUDCA-activation of ERK1/2 MAP kinase and PI3 kinase could be a mechanism to act on Bax. All together, our data fit very well with mitochondria-controlled bile acid-induced apoptosis in primary hepatocytes and provide more information about the link between TUDCA-activated survival pathways and mitochondria.
Interestingly, our data are in contrast to a recent study describing that the protection of TUDCA against TLCS-induced apoptosis is p38 MAP kinase-, ERK1/2 MAP kinase- and PI3 kinase-independent, and depends on inhibition of Fas trafficking and caspase-8 activation.(41) The authors suggest that TUDCA does inhibit TLCS-induced apoptosis upstream of caspase-8 activation. However, they also suggest that TUDCA inhibits TLCS-triggered mitochondrial ROS formation (41), which is needed for Fas trafficking.(39) Therefore, TUDCA may inhibit TLCS-induced apoptosis in a mitochondria-controlled manner. Alternatively, GCDCA and TLCS may induce apoptosis via different mechanisms, which could explain the discrepancies between the protective mechanisms of TUDCA against these bile acids.
The activation of the p38 MAP kinase pathway by TUDCA in order to inhibit GCDCA-induced stress in rat hepatocytes is in line with the activation of p38 MAP kinases during other forms of environmental stress. (14) Several reports describe the involvement of these kinases in mRNA stabilization (42). Since we demonstrated that the protective effect of TUDCA is at a post-transcriptional level, this mechanism could be present in primary rat hepatocytes as well.
Although, the NF-κB pathway is involved in protection against cytokine-induced stress in primary rat hepatocytes (11) (12), we did not find evidence that the NF-κB pathway is involved in the protective action of TUDCA against GCDCA-induced apoptosis. Bile acids do not directly activate NF-κB (13). Moreover, inhibition of the NF-κB pathway did not change the protective action of TUDCA.

In summary, we have shown that the anti-apoptotic effect of TUDCA against GCDCA-induced apoptosis in primary rat hepatocytes is independent of caspase-8 inhibition, but is due to activation of p38, ERK MAP kinases and PI3 kinase survival pathways. Furthermore, we have demonstrated that TUDCA protects against GCDCA-induced mitochondrial injury in

primary rat hepatocytes. Our data provide more information about the mechanism of action of UDCA in cholestatic liver diseases.

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Reference List

1. Poupon RE, Lindor KD, Cauch-Dudek K, Dickson ER, Poupon R, Heathcote EJ. Combined analysis of randomized controlled trials of ursodeoxycholic acid in primary biliary cirrhosis. Gastroenterology 1997; 113(3):884-890.
2. Paumgartner G, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. Hepatology 2002; 36(3):525-531.
3. Guldutuna S, Zimmer G, Imhof M, Bhatti S, You T, Leuschner U. Molecular aspects of membrane stabilization by ursodeoxycholate [see comment]. Gastroenterology 1993; 104(6):1736-1744.
4. Van Nieuwkerk CM, Elferink RP, Groen AK, Ottenhoff R, Tytgat GN, Dingemans KP et al. Effects of Ursodeoxycholate and cholate feeding on liver disease in FVB mice with a disrupted mdr2 P-glycoprotein gene. Gastroenterology 1996; 111(1):165-171.
5. Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G et al. Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. Hepatology 2001; 33(5):1206-1216.
6. Kurz AK, Graf D, Schmitt M, Vom DS, Haussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. Gastroenterology 2001; 121(2):407-419.
7. Botla R, Spivey JR, Aguilar H, Bronk SF, Gores GJ. Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. J Pharmacol Exp Ther 1995; 272(2):930-938.
8. Rodrigues CM, Fan G, Ma X, Kren BT, Steer CJ. A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation. J Clin Invest 1998; 101(12):2790-2799.
9. Rodrigues CM, Ma X, Linehan-Stieers C, Fan G, Kren BT, Steer CJ. Ursodeoxycholic acid prevents cytochrome c release in apoptosis by inhibiting mitochondrial membrane depolarization and channel formation. Cell Death Differ 1999; 6(9):842-854.
10. Rodrigues CM, Sola S, Sharpe JC, Moura JJ, Steer CJ. Tauroursodeoxycholic acid prevents bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. Biochemistry 2003; 42(10):3070-3080.
11. Schoemaker MH, Ros JE, Homan M, Trautwein C, Liston P, Poelstra K et al. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. J Hepatol 2002; 36(6):742-750.
12. Hatano E, Bennett BL, Manning AM, Qian T, Lemasters JJ, Brenner DA. NF-kappaB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis. Gastroenterology 2001; 120(5):1251-1262.
13. Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Trautwein C, Van Goor H et al. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to NF-kB activation. J.Hepatol. 39(2), 153-161. 2003.
14. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002; 298(5600):1911-1912.
15. Qiao L, Yacoub A, Studer E, Gupta S, Pei XY, Grant S et al. Inhibition of the MAPK and PI3K pathways enhances UDCA-induced apoptosis in primary rodent hepatocytes. Hepatology 2002; 35(4):779-789.

16. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999; 13(22):2905-2927.
17. Perwaiz S, Tuchweber B, Mignault D, Gilat T, Yousef IM. Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry. J Lipid Res 2001; 42(1):114-119.
18. Combes B, Carithers RL, Jr., Maddrey WC, Munoz S, Garcia-Tsao G, Bonner GF et al. Biliary bile acids in primary biliary cirrhosis: effect of ursodeoxycholic acid. Hepatology 1999; 29(6):1649-1654.
19. Invernizzi P, Setchell KD, Crosignani A, Battezzati PM, Larghi A, O'Connell NC et al. Differences in the metabolism and disposition of ursodeoxycholic acid and of its taurine-conjugated species in patients with primary biliary cirrhosis. Hepatology 1999; 29(2):320-327.
20. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. Hepatology 1990; 12(3 Pt 1):511-518.
21. Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL et al. Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology 2002; 35(3):589-596.
22. Kullak-Ublick GA, Ismair MG, Kubitz R, Schmitt M, Haussinger D, Stieger B et al. Stable expression and functional characterization of a Na+-taurocholate cotransporting green fluorescent protein in human hepatoblastoma HepG2 cells. Cytotechnology 34, 1-9. 2000.
23. Iimuro Y, Nishiura T, Hellerbrand C, Behrns KE, Schoonhoven R, Grisham JW et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration [published erratum appears in J Clin Invest 1998 Apr 1;101(7):1541]. J Clin Invest 1998; 101(4):802-811.
24. Mills CO, Milkiewicz P, Muller M, Roma MG, Havinga R, Coleman R et al. Different pathways of canalicular secretion of sulfated and non-sulfated fluorescent bile acids: a study in isolated hepatocyte couplets and TR- rats. J Hepatol 1999; 31(4):678-684.
25. Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R et al. Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. Mol Biol Cell 2001; 12(9):2629-2645.
26. Vos TA, Gouw AS, Klok PA, Havinga R, Van Goor H, Huitema S et al. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. Gastroenterology 1997; 113(4):1323-1333.
27. Miyoshi H, Rust C, Roberts PJ, Burgart LJ, Gores GJ. Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 1999; 117(3):669-677.
28. Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA et al. Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. J Clin Invest 1999; 103(1):137-145.
29. Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El Deiry W et al. The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. J Biol Chem 2001; 276(42):38610-38618.
30. Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. Hepatology 2001; 33(4):776-782.
31. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998; 17(8):2215-2223.
32. Takikawa Y, Miyoshi H, Rust C, Roberts P, Siegel R, Mandal PK et al. The bile acid-activated phosphatidylinositol 3-kinase pathway inhibits Fas apoptosis upstream of bid in rodent hepatocytes. Gastroenterology 2001; 120(7):1810-1817.
33. Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ. BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. J Biol Chem 1997; 272(39):24101-24104.
34. Muslin AJ, Tanner JW, Allen PM, Shaw AS. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell 1996; 84(6):889-897.
35. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998; 282(5392):1318-1321.
36. Tsuruta F, Masuyama N, Gotoh Y. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. J Biol Chem 2002; 277(16):14040-14047.
37. Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P et al. Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. Hepatology 2002; 35(2):307-314.
38. Yoon JH, Higuchi H, Werneburg NW, Kaufmann SH, Gores GJ. Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. Gastroenterology 2002; 122(4):985-993.
39. Reinehr R, Graf D, Haussinger D. Bile salt-induced hepatocyte apoptosis involves epidermal growth factor receptor-dependent CD95 tyrosine phosphorylation. Gastroenterology 2003; 125(3):839-853.

40. Zhang XD, Borrow JM, Zhang XY, Nguyen T, Hersey P. Activation of ERK1/2 protects melanoma cells from TRAIL-induced apoptosis by inhibiting Smac/DIABLO release from mitochondria. Oncogene 2003; 22(19):2869-2881.
41. Graf D, Kurz AK, Fischer R, Reinehr R, Haussinger D. Taurolithocholic acid-3 sulfate induces CD95 trafficking and apoptosis in a c-Jun N-terminal kinase-dependent manner. Gastroenterology 2002; 122(5):1411-1427.
42. Reunanen N, Li SP, Ahonen M, Foschi M, Han J, Kahari VM. Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. J Biol Chem 2002; 277(35):32360-32368.